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IMMUNOLOGICAL APPROACH TO THE IDENTIFICATION AND DEVELOPMENT OF VACCINES TO VARIOUS TOXINS

ANNUAL REPORT

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Murine monoclonal anti-saxitoxin and anti-ricin antibodies capable of protecting against the in vitro toxicity of saxitoxin and ricin, respectively, have been generated. These protective anti-toxin (Ab_1) antibodies are being used to induce the production of internal image monoclonal anti-idiotypic antibodies (Ab_2) which may serve as vaccines in the induction of active and protective immunity against these toxins. The murine responses to synthetic peptides homologous to immunogenic amino acid sequences of the ricin A and B chains have also been investigated either singly or in combination with the aim of developing a subunit vaccine against ricin in vivo toxicity.

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FOREWORD

In the conducting of the research described in the report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources - National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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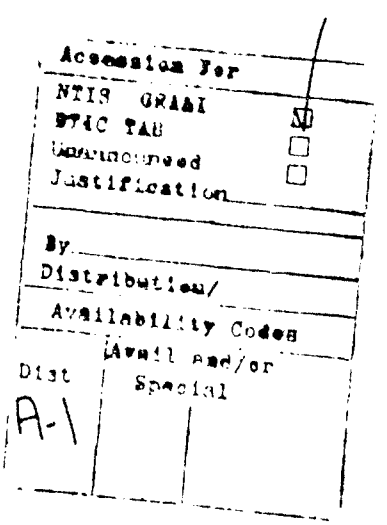

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I. INTRODUCTION

The sodium channel blocker **saxitoxin** (SXT) and the protein synthesis inhibitor **ricin** are highly toxic compounds. The nonproteinaceous nature of SXT, its small molecular weight, and the extreme toxicity of these two compounds have rendered the development of vaccines against their toxicity difficult. During the first year of this contract, we have attempted to develop anti-idiotype (anti-Id) based vaccines for the induction of active and protective immunity against the toxicity of SXT and ricin. Our approach involves the generation of monoclonal anti-toxin (Ab_1) antibodies of high binding affinity which are specific for the toxins and which protect against their <u>in vivo</u> toxicity. These monoclonal Ab_1 will then be used to generate monoclonal anti-antibodies (anti-Id or Ab_2). Monoclonal Ab_2 selected for their ability to mimic the nominal antigen (toxins) will be assessed for their ability to induce an active and effective immunity against the toxins.

Since ricin is a protein composed of two polypeptide chains (chains A and B) whose primary amino acid sequences are known, we also proposed to investigate the feasibility of using synthetic peptides homologous to antigenic sequences of the A and/or B chains either singly or in combination as a subunit vaccine against ricin toxicity.

This annual report describes the generation of monoclonal antibodies (mAbs) against SXT, and against ricin using whole ricin, isolated A and B chains, and ricin synthetic peptides as immunogens. The anti-ricin immune responses in BALB/C mice immunized with synthetic peptides homologous to sequences of the ricin A and B chains either singly or in combination are also discussed.

II. RESULTS

A. SAXITOXIN

Within the last year, fusions of spleen cells from mice immunized with SXT conjugated to keyhole limpet hemocyanin (KLH) using either formaldehyde or N-succinimidyl 3-2 (pyridyldithio) propionate (SPDP) as linkers have been performed. The fusion procedure was done as described in previous reports (also see reference 1). A total of approximately 1200 hybrids have been screened from two fusions of spleen cells from mice immunized with SXT-formaldehyde-KLH and three fusions of spleen cells from mice immunized with SXT-SPDP-KLH (data not shown). Up to date, although specific anti-SXT mAbs have been isolated with ease, we have not been able to generate anti-SXT mAbs with higher binding affinities than the ones we have isolated previously (2).

B. RICIN

1. Monoclonal Antibodies Raised Against Native Ricin: A major emphasis of the current contract involves the generation of mAbs specific for ricin which can protect against its toxicity. BALB/C mice were immunized with whole ricin molecule, purified ricin A chain or purified ricin B chain (Sigma Chemical Co., St. Louis, MO). Each mouse received every two weeks intraperitoneal injections of 10 ug of the antigen in Freund's compete adjuvant (FCA), followed by antigen in Freund's incomplete adjuvant (FIA) for the second booster injection and in phosphate buffered saline (PBS, pH 7.2) for subsequent injections. The mice were bled before each immunization, and their sera tested for reactivity in enzyme-linked immunosorbent assays (ELISA). Microtiter wells were coated with a pretitrated amount of whole ricin (10 ug/ml), or ricin chain A (10 ug/ml), or ricin chain B (10 ug/ml) overnight at $4^{o}C$. The wells were washed with PBS supplemented with 5% normal goat serum (PBS/NGS), and blocked with PBS/NGS for 1 hr at $37^{o}C$. The wells were then washed, and 0.05 ml of serial dilutions of preimmune or immune sera were added. After 1hr of incubation at $37^{o}C$ and washing, 0.05 ml of a 1:2500 dilution of goat anti-mouse Ig conjugated to horseradish peroxidase (HRP, Fisher Scientific, Orangeburg, NY) were added. Reactivity was detected by addition of the substrate ABTS (2,2-azino-bis-[3-ethyl-benzthiazoline-6-sulfonate], Sigma Chemical Co.). Positive reactivity is defined as optical density (OD_{410nm}) greater than 3 times that of the negative control (preimmune sera). Positive controls used were culture supernatants from two hybridomas, TFTA-1 anti-ricin A chain and TFTB-1 anti-ricin B chain (American Type Culture Collection, Rockville, MD). Culture supernatant from HD11 hybrids producing an anti-T-2 mycotoxin IgG_1 mAb served as a negative control (3). Six fusions have been performed using mice immunized with whole ricin, three fusions with ricin chain A-immune spleen cells, and two fusions with ricin B chain-immune spleen cells. Positive fusion hybrid supernatants were selected and further tested in an inhibition ELISA. Positive

culture supernatants were incubated with an equivalent volume of various concentrations of inhibitors (whole ricin, ricin chains A or B) for 1 hr at 37°C. After incubation, the mixtures were added to wells coated with the immunizing antigen, and the ELISA was done as described above. Positive culture supernatants whose binding was inhibited by greater than 30% were further selected for the EL-4 in vitro protection assay. The ricin toxicity on EL-4 cells was first determined by using different concentrations of ricin. EL-4 cells obtained from Dr. J. F. Hewetson (Pathophysiology Division, USAMRIID, Fort Detrick, Frederick, MD) were washed and resuspended at a cell density of $2x10^6$ cells/ml in Dubbelco MEM (DMEM) supplemented with 10% fetal bovine serum (FBS). Triplicates of 0.05 ml containing $1x10^5$ were added to 96-well flat bottom tissue culture plates. Different concentrations of ricin (0.05 ml) in leucine-free DMEM were added, and the wells incubated at 37°C overnight. After incubation, 0.05 ml of DMEM containing 1.0 uCi of [^{3}H]leucine were added to the wells which were incubated for an additional 4 hr at 37°C. The cells were then harvested and processed for scintillation counting. The concentration of ricin providing approximately 50% inhibition of [^{3}H]leucine uptake by EL-4 cells in our assay system is 6.0 ng/ml, a concentration now routinely use in our laboratory for the assessment of antibody protection. In this assay, the proper ricin concentration is incubated with an equivalent volume of anti-ricin antibodies for 1 hr at 37°C. The mixtures are then added to the EL-4 containing wells to give a final ricin concentration of 6.0 ng/ml, and the assay performed as described above.

Thus far, six hybrid clones have been derived from fusions of whole ricin immune spleen cells, whereas one hybrid was isolated from ricin A chain fusions. The results of the binding and inhibition ELISA, and of the EL-4 in vitro assays are presented in Table 1. All six mAbs are of the IgM isotype. They reacted strongly in ELISA using whole ricin- and ricin A chain-coated microtiter wells. E4, E7 and D6 mAbs reacted to a lesser extent with B chain-coated wells, whereas A-1, 3F2 and WR1C4 mAbs did not. The binding of A-1 and 3F2 mAbs to whole ricin-coated wells were inhibited effectively by ricin A chain, but not by whole ricin or ricin B chain. The highest concentration of inhibitors used routinely is 200 ug/ml based on the assumption that mAbs whose binding are not inhibited by this concentration would in all likelihood not possess high binding affinity. Whether A-1 and 3F2 mAbs binding would be inhibited by higher concentrations of whole ricin or ricin B chain remain to be determined. The binding of the other four hybrids (E4, E7, D6 and WR1C4) were inhibited most strongly by ricin A chain followed by whole ricin, and to a much lesser degree by ricin B chain. Thus, immunization with whole ricin and screening of the hybrid culture supernatants in ELISA using whole ricin-coated wells appears to yield anti-ricin mAbs with specificity for ricin A chain. E4, E7, D6, 3F2 and WR1C4 mAbs protected against the in vitro toxicity of ricin in EL-4 assays, whereas A-1 mAb only gave approximately 29% protection against ricin toxicity (Table 1). Purified mAbs from these four hybrids have been prepared. They will be conjugated to KLH and alum precipitated for anti-Id produc-

tion. One mAb (2H11, IgG_1) was isolated from fusions of ricin A chain-immune spleen cells. 2H11 mAb reacted with whole ricin- and ricin A chain-coated wells, but not with ricin B chain-coated wells. Its binding to ricin A chain-coated wells was inhibited by approximately 77.7%, 43.3% and 24.1% by 200 ug/ml of chain A, whole ricin and chain B, respectively. It is not known why 2H11 reactivity was inhibited by purified B chain. However, one likely possibility is contamination of the purified B chain preparation with ricin A chain. In EL-4 assays, 2H11 protected against ricin toxicity by approximately 36.3% (Table 1).

2. Monoclonal Antibodies Raised Against Ricin A Chain Synthetic Peptide: A synthetic peptide homologous to a solvent-exposed alpha-helical amino acid sequence of the ricin A chain (**A-18**, Figure 1 and see below) was synthesized, and used to immunize BALB/C mice. Two fusions have been done with A-18 immune spleen cells. Three mAbs designated 1C2 (IgG_{2a}), 1D6 (IgG_{2b}) and 4F (IgG_1) were isolated. They were specific in their binding to whole ricin and ricin chain A, but did not react with ricin B chain (Table 1). Their binding was inhibited by 200 ug/ml of ricin A chain, however no significant inhibition was observed with whole ricin or ricin B chain. EL-4 assays with these mAbs showed only partial protection against ricin toxicity ranging from 25% to 45%.

3. Murine Immune Responses Against Synthetic Peptides Homologous to Ricin A and B Chains: In addition to our attempt to generate anti-Id which may serve as vaccine against ricin toxicity, we also proposed to develop synthetic peptide-based subunit vaccines. The rationale for peptide selection has been discussed in details previously. Briefly, peptides selected for synthesis were based on properties such as hydrophobicity/hydrophylicity indices, alpha-helical structures, beta-turns, galactose-binding domains of the ricin B chain, and exposure to solvent or potential accessibility for immune recognition. Two amino acid sequences homologous to the ricin A chain, designated **A-18 and A-95** were selected because they represent two solvent-exposed alpha-helical structures. Their amino acid sequences and other characteristics are shown in figures 1 and 2, respectively. Two sequences were also selected from the ricin B chain. Designated **B-18 and B-230**, they encompass the galactose-binding domains of the ricin molecule (Figures 3 and 4). Three (A-18, A-95 and B-230) of these four peptides were synthesized in our laboratory as free peptides which were then conjugated to KLH for immunization purpose. The fourth peptide (B-18) was synthesized on a backbone of branching lysine core as described by Posnett et al. (4), and referred to as multiple antigenic peptide (**MAP**, Figure 5). Peptides synthesized in this fashion has been demonstrated to render the need for carrier proteins obsolete in generating high tittered anti-peptide and anti-native antibodies. Moreover, the possibility exists in the synthesis of MAP with multiple sets of peptide arms, or the synthesis of polymers containing two or more different peptides. This last property may be important in the development of effective vaccines in instances where there is a requirement for more than one protective immunogenic epitopes.

Groups of BALB/C mice were immunized with the synthetic peptides in various adjuvants as shown below:

A-18 Peptide (6 mice/group)	**A-95 Peptide** (5 mice/group)
1. A-18 in alum	1. A-95 in alum
2. A-18-KLH in alum	2. A-95-KLH in alum
3. A-18-KLH in FCA	3. A-95-KLH in FCA
	4. A-95-KLH in FIA

B-230 Peptide	**B-18-MAP Peptide**
1. B-230 in alum	1. B-18-MAP in PBS
2. B-230 in FCA	2. B-18-MAP in alum
3. B-230-KLH in alum	3. B-18-MAP in FCA
4. B-230-KLH in FCA	4. B-18-MAP in FIA

Each mouse received 10 ug of peptides A-18 or A-95, or 50 ug of peptides of B-230 or B-18-MAP intraperitoneally per immunization every other week. The reason for increasing the dose of the latter two peptides is the low responses obtained with 10 ug of A-18 and A-95 peptides (see below). However, it became apparent that the higher dose did not result in an enhanced antibody response, but that peptide B-18 synthesized as MAP will induce a significantly stronger anti-ricin immune response.

a) Immune Responses to Ricin A-18 Peptide: The binding ELISA results of mice immunized with A-18 peptide, encompassing an alpha-helical and solvent-exposed sequence of the ricin A chain, using A-18-, whole ricin- and ricin A chain-coated wells are shown in Table 2. The majority of the mice immunized with either unconjugated A-18 peptide, or with A-18-KLH in FCA or FIA developed detectable anti-ricin antibodies following the 2^{nd} or 3^{rd} injection. The sera reacted not only with the immunizing peptide, but also with whole ricin and ricin A chain. The serum titer of the reactivity against A chain (ranging from dilutions of 1:40 to 1:1280) appeared to be somewhat higher than those against A-18 peptide (1:20 to 1:160) and those against whole ricin (1:20 to 1:40). No significant reactivity was observed with ricin B chain (data not shown). With few exceptions, the serum titers of all three groups of mice did not increase significantly with booster injections and remained rather modest up to the 5^{th} or 6^{th} injection. No obvious differences was obtained with A-18 peptide in its free from or conjugated to KLH emulsified in alum or in FCA.

b) Immune Responses to Ricin A-95 Peptide: The serum reactivity of mice immunized with ricin A-95 peptide which represents another alpha-helical and hydrophylic sequence of the A chain are shown in Table 3. Unlike peptide A-18, conjugation of A-95 to KLH and immunization either as alum precipitate or in FCA did not result in a significant anti-peptide or anti-ricin antibody response even following the 6^{th} immunization. On the other hand,

unconjugated A-95 peptide injected in the presence of alum or FCA induced a detectable anti-peptide and anti-ricin reactivity after 2 to 3 injections. The reason for this is unclear, however it is possible that KLH conjugation may render A-95 nonimmunogenic. As is the case with A-18 peptide, A-95 immune sera reacted with the immunizing peptide, ricin A chain and whole ricin, but not with ricin B chain. The antibody titers in all groups did not increase significantly after the 6^{th} immunization. Moreover, different adjuvants did not appear to influence the antibody titer.

c) Immune Responses to Ricin B-230 Peptide: Groups of mice were also immunized with B-230 peptide which encompasses one of the two galactose-binding domains of the ricin B chain. The results of ELISA reactivity of B-230 immunization, given in Table 4, are somewhat confusing and disappointing. When unconjugated, B-230 peptide in alum or in FCA induced primarily an anti-peptide response which was apparent only following the 3^{rd} or 4^{th} immunization. No reactivity was observed with the native ricin B chain, whereas only minimal reactivity was obtained against whole ricin after the 5^{th} injection. KLH-conjugated B-230 in alum induced an anti-whole response only after the 5^{th} injection, however no reactivity was observed with the homologous peptide (B-230) or with ricin B chain. Immunization of B-230-KLH in FCA resulted in a strict anti-B-230 antibody response with no detectable anti-native response.

In a set of experiments designed to assess the effects of A-18, A-95 and B-230 immunizations against the *in vitro* toxicity of ricin, 3 mice from each group were given an additional booster injection of the corresponding peptide in the appropriate adjuvant. Two weeks thereafter, the sera were tested in EL-4 assays as described above. No significant protection was observed (data not shown).

We had previously titrated the *in vivo* toxicity of ricin in BALB/C mice by injecting intraperitoneally (i.p.) or intramuscularly (i.m.) groups of 5 mice each with various doses of ricin. In our hands, the ricin LD_{50} in BALB/C mice appeared to be between 0.1 ug/mouse and 0.5 ug/mouse for both routes of administration (Table 5). All the mice that received peptide booster injections above, and a group of control unimmunized mice were challenged with 1.0 ug/mouse of ricin i.m. in the right thigh. All mice succumb to ricin toxicity within 18 hr to 36 hr following ricin administration.

In summary, ricin A-18 peptide either free or KLH conjugated, and ricin A-95 peptide free but not conjugated to KLH were capable of inducing anti-peptide and anti-native ricin antibody responses. However, the titer of the responses remained rather low even following multiple injections. Immunization with B-230 peptide elicited only a low anti-peptide response without significant anti-native reactivity. These 3 ricin peptides injected as described above did not elicit detectable protective immune responses against ricin *in vitro* and *in vivo* toxicity. Various combinations of these

3 peptides are currently being assessed either as singly or in combination for their potential to generate a high tittered and protective anti-toxin antibody response.

d) Immune Responses to Ricin B-18-MAP: As mentioned previously, a synthetic peptide homologous to one of the galactose-binding domain of ricin B chain (residues 18-48) was synthesized on a backbone of lysine residues, termed MAP (**B-18-MAP**). Four groups of mice each were immunized as outlined above. Their sera were tested for reactivity with B-18-MAP-, whole ricin-, ricin A- or ricin B chain-coated wells (Table 6). Unlike peptides A-18, A-95 and B-230, which requires at least 2 to 3 injections before detection of a low antibody response, ricin B-18-MAP administered in PBS or in alum induced a detectable albeit low antibody response following the 1^{rst} immunization. The anti-peptide and anti-native antibody responses increased dramatically following subsequent injections. Two weeks after the 4th injection, the antibody titers reached levels of 1:1,000 to greater than 1:10,000 dilutions, which were not achieved with 5 or 6 injections of the other 3 peptides. The two groups of mice immunized with B-18-MAP in FCA or in FIA gave low responses only following the 2nd or 3rd injection. The reason for this is not clear, however, it is noteworthy that B-18-MAP peptide did not emulsify well in these adjuvants because of their low solubility. Nevertheless, after the 4th injection, these two groups of mice developed titers comparable to if not higher tan those of the PBS and alum groups. The immune response to B-18-MAP was specific in that no significant reactivity was obtained against ricin A chain (data not shown). Selected sera also tested negative against A-18, A-95 and B-230 peptides.

Sera from two mice in each group were tested in EL-4 protection assays (Table 7). Sera of two mice from each of group A (PBS) and B (alum) did not protect against the in vitro toxicity of ricin at a serum dilution of 1:20. On the other hand, those of groups C (FCA) and D (FIA) showed significant protection.

These results suggest that B-18-MAP peptide induces a significantly higher anti-peptide and anti-native antibody responses than could be obtained with A-18, A-95 and B-230 ricin peptides. The EL-4 assay results also suggest that the antibody responses obtained with B-18-MAP immunization may be associated with protection against ricin toxicity. It is not known at the present time whether this enhanced antigenicity and associated protection were due to the synthesis of the peptide on the MAP system or to the inherent immunogenic property of the B-18 peptide sequence itself. We have recently synthesized A-18 and B-230 peptides on MAP in order to further investigate this issue.

4. Rabbit Polyclonal Anti-Ricin Antibodies:

Two New Zealand White rabbits (X-839 and X-840) were immunized i.m. with 50 ug/rabbit of ricin in FCA for the 1rst injection, followed by antigen in FIA for the 2nd injection, and in PBS for subsequent injections. After 4 immunizations given two weeks

apart, the rabbits were bled and their sera tested for binding in ELISA and for protection in EL-4 assays. The results indicate that after 4 injections, the rabbits developed a good anti-ricin antibody titer (1:5,000 to 1:10,000). The rabbit IgG anti-ricin was purified by protein A-agarose affinity chromatography and tested for protection in EL-4 assays. The results of rabbit X-840 anti-ricin IgG in EL 4 protection assays are shown in Table 8. The purified rabbit IgG showed significant protection against the *in vitro* toxicity of ricin, whereas the preimmune IgG did not. Similar results were obtained with rabbit X-839 anti-ricin IgG (data not shown).

III. SUMMARY

We have generated a panel of monoclonal anti-ricin antibodies with diverse specificities, some of which were capable of protecting against the *in vitro* toxicity of ricin as determined in the EL-4 assays. Protective rabbit polyclonal anti-ricin IgG have also been generated and purified. Both monoclonal murine and rabbit polyclonal anti-ricin antibodies are being utilized to generate in mice anti-idiotypic antibodies which may exhibit the internal image of ricin, and which may serve as antibody-based vaccine against the *in vivo* toxicity of ricin.

The immunogenicity of four peptides homologous to various amino acid sequences of the A or B chain of ricin have been investigated. The results to date suggest that at least B-18 peptide synthesized on the MAP system may provide an enhanced and protective immune response against ricin toxicity.

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Table 1

Characterization of Monoclonal Anti-Ricin Antibodies

MAbs	Immunogen	Isotype	Reactivity with			%I by			EL-4
			Whole	A	B	Whole	A	B	
A-1	Whole	IgM	+	+	-	2.2	82.5	2.4	28.8
10G7(E4)	ricin	"	+	+	±	35.6	70.1	15.6	100.0
10G7(E7)	"	"	+	+	±	24.9	90.5	9.2	98.2
10G7(D6)	"	"	+	+	±	40.6	55.4	11.3	96.1
3F2	"	"	+	+	-	9.6	48.0	0	84.7
WR1C4	"	"	+	+	-	55.2	58.5	5.1	91.5
2H11	A chain	IgG1	+	+	-	43.3	77.7	24.1	36.3
1C2	A-18	IgG2a	+	+	-	2.6	77.8	2.5	45.2
1D6	"	IgG2b	+	+	-	3.7	93.4	0	29.5
4F	"	IgG1	+	+	-	6.2	58.0	1.2	37.8

[a]Positive defined as OD 3x> control OD.
[b]Negative defined as OD <2x control OD.
[c]± defined as OD between 2 to 3 times that of control OD.
[d]Mean % protection defined as [mean % inhibition of labeled leucine uptake without mAb]-[mean % uptake with mAb]/[mean % uptake without mAb] x 100. The EL4 assays were done in triplicate.

Table 2

REACTIVITY OF MICE IMMUNIZED WITH RICIN A18 PEPTIDE

Mouse No.	A18-coated wells				Whole-coated wells				A Chain-coated wells			
	3°	4°	5°	6°	3°	4°	5°	6°	3°	4°	5°	6°
100	40[a]	80	40	80	10	80	20	40	160	40	160	160
122	60	80	40	20	80	80	20	20	160	80	80	80
101 Alum	80	80	80	160	40	160	20	20	80	160	160	640
102	40	40	20	40	320	80	20	20	160	80	80	80
110	80	80	20	20	80	160	10	20	80	80	80	160
120	320	320	40	40	80	320	40	40	160	160	160	80
200	80	40	20	20	160	80	40	40	80	160	640	1280
201	40	160	20	20	40	80	40	40	160	80	320	320
210 KLH	80	80	10	10	40	80	20	20	160	80	40	40
202 Alum	80	80	10	10	40	40	20	20	80	80	40	40
220	80	160	40	40	80	80	20	20	160	80	80	80
222	-	160	20	20	80	80	40	40	80	80	80	80
310	20	40	20	20	40	40	10	10	40	80	40	40
302 KLH	20	40	10	10	40	40	10	10	80	80	20	40
301 FCA	60	160	80	80	40	80	20	20	160	160	160	160
320	40	40	10	10	40	40	10	20	80	80	40	80
300	80	80	40	80	80	160	20	20	160	160	160	160
322	80	160	320	320	40	160	40	40	80	160	1280	320

[a]Reciprocal dilution of serum giving positive reaction (defined as at least three times above the preimmune serum)

Table 3

REACTIVITY OF MICE IMMUNIZED WITH RICIN A95 PEPTIDE

Mouse No.		A95-coated wells				Ricin coated-wells				Ricin A-coated wells			
		2°	3°	4°	5°	2°	3°	4°	5°	2°	3°	4°	5°
900		40[a]	40	80	160	80	40	40	40	80	40	160	160
901		40	40	80	40	40	20	20	40	40	20	160	80
910	Alum	10	20	80	80	40	40	40	40	20	10	80	80
920		40	20	80	80	80	40	40	20	80	80	160	40
902		Mouse Died											
800		0	0	0	0	10	0	0	0	20	0	0	0
801	KLH	0	0	0	0	0	0	0	0	0	20	20	0
802	Alum	0	0	0	20	10	0	0	0	0	20	0	0
810		0	0	0	20	10	0	0	10	20	10	10	0
820		0	0	0	0	20	0	0	0	20	20	20	0
10-00		0	0	0	0	10	0	0	0	20	20	20	0
10-01	KLH	0	0	0	0	20	0	0	0	10	0	0	0
10-10	FCA	0	0	0	0	10	0	0	0	10	0	0	0
10-20		0	0	20	0	10	0	0	0	20	0	0	0
10-02		0	20	10	0	20	0	0	0	10	0	0	0
11-00		10	10	40	40	40	20	40	40	80	40	80	40
11-01		20	20	80	40	80	20	40	40	80	80	160	80
11-10	FCA	20	10	40	80	40	20	40	40	40	20	80	80
11-20		0	0	20	40	20	40	40	40	20	20	80	40
11-02		20	10	80	40	80	40	40	40	160	80	160	80

See legend of Table 2.

Table 4

REACTIVITY OF MICE IMMUNIZED WITH RICIN B230 PEPTIDE

Mouse No.		B230-coated wells					Whole Ricin-coated wells					B-chain-coated wells				
		1°	2°	3°	4°	5°	1°	2°	3°	4°	5°	1°	2°	3°	4°	5°
401		0[a]	0	0	0	0	-	-	0	0	80	-	-	10	0	0
402	KLH	0	0	0	0	0	-	-	40	0	160	-	-	0	0	0
410	Alum	0	0	0	0	0	-	-	0	0	80	-	0	0	0	0
420		0	0	0	0	0	-	40	0	0	160	-	0	0	0	0
422		0	20	0	10	0	-		80	0	160	-	0	0	0	0
500(D)		0	80	80	160	80	-	40	0	-	320	-	0	0	0	0
502		0	0	20	80	320	-	-	40	0	40	-	-	40	0	0
510	Alum	0	0	0	80	160	-	-	40	0	40	-	-	40	0	0
520		0	0	80	40	80	-	-	20	0	10	-	20	0	0	0
522		0	0	20		320	-	-	20	0	40	-	20	0	0	0
600		0	0	0	160	160	-	-	20	0	0	-	20	0	0	0
601	KLH	0	0	0	80	640	-	-	20	0	0	-	20	0	20	0
620	FCA	0	0	20	40	160	-	-	20	0	10	-	20	0	0	0
622		0	0	0	80	160	-	-	20	0	0	-	0	0	20	0
700		0	0	0	20	80	-	-	0	0	40	-	0	0	0	0
701	FCA	0	0	0	20	320	-	-	10	0	80	-	0	0	0	0
702		0	0	0	40	160	-	-	0	0	80	-	0	0	0	0
710		0	0	0	20	320	-	-	80	0	160	-	80	0	0	0

[a]Reciprocal dilution of immune sera giving OD_{410nm} at least three times above that of the preimmune sera.

Table 5

RICIN *IN VIVO* LD_{50} IN MICE

Intraperitoneal		Intramuscular	
Ricin (ug/mouse)	Survival Ratio[a]	Ricin (ug/mouse)	Survival Ratio
5.0	0/5	2.0	0/5
1.0	0/5	0.5	0/5
0.5	0/5	0.1	5/5
0.1	5/5		

[a]Number of mice dead/total number of mice.

Table 6

Reactivity of Mice Immunized with Ricin B-18-MAP

Mouse No.	Reactivity with							
	Whole Ricin				B Chain			
	1°*	2°	3°	4°	1°	2°	3°	4°
in PBS:								
A-00	40[a]	80	80	160	80	320	160	640
A-10	0	160	160	320	0	40	160	1280
A-02	20	40	320	1280	40	320	1280	10000
A-11	20	80	640	1280	20	640	1280	10000
A-20	20	ND	640	640	80	ND	640	1280
in Alum:								
B-00	10	160	640	10000	20	320	2560	>10000
B-10	20	320	640	>10000	20	320	640	>10000
B-11	40	640	640	5120	40	320	640	>10000
B-02	10	320	1280	5120	20	640	1280	>10000
B-20	20	160	320	2560	40	640	640	>10000
in FCA:								
C-00	0	0	20	640	0	20	320	1280
C-10	0	40	160	1280	0	20	640	>10000
C-11	0	40	320	1280	20	80	640	>10000
C-02	0	20	160	1280	0	20	320	10000
C-20	0	20	20	640	0	20	40	>10000
in FIA:								
D-00	0	0	80	160	0	0	640	10000
D-10	20	20	80	>10000	40	40	1280	>10000
D-11	0	20	160	640	0	40	640	>10000
D-20	0	ND	160	1280	0	ND	640	>10000

*Two-week bleed post number of injections indicated.
[a]Reciprocal dilutions giving positive reactivity defined as OD>3x preimmune OD.

Table 7

Protection Against the In Vitro Cytotoxicity of Ricin by Sera from Mice Immunized with MAP.B-18

Mouse No.	Sera dilution	Adjuvant	% Inhibition of [^{3}H]leucine uptake	% Protection
None	None	None	45.3[a]	
NMS	1:20	None	51.2	0[b]
A-02	1:20	PBS	49.5	0
A-20	"	"	52.1	0
B-02	1:20	Alum	44.7	1.3
B-20	"	"	43.1	4.9
C-10	1:20	FCA	7.2	84.1
C-11	"	"	27.2	39.9
D-10	1:20	FIA	10.9	75.9
D-11	"	"	1.8	96.0

[a]Mean % inhibition of triplicate cultures.
[b]Mean percent protection in triplicate EL-4 assays

Table 8

Rabbit Anti-Ricin IgG Protect Against Ricin *In Vitro* Tocxicity

Rabbit X-840 IgG (ug/ml)	EL4 Cells Cultured (cpm)		%I[a]
	Without Ricin	With Ricin[b]	
None	101,512 ± 4,872[c]	48,521 ± 3,267	52.2
Anti-Ricin IgG			
(20.0)	98,045 ± 5,001	102,156 ± 4,913	0
(0.2)	103,527 ± 7,109	88,937 ± 3,296	14.1
(0.02)	95,842 ± 4,901	65,912 ± 3,902	31.2
(0.005)	99,390 ± 3,956	54,937 ± 2,917	44.7
Preimmune IgG			
(20.0)	95,684 ± 4,178	50,034 ± 3,290	47.7
(10.0)	101,256 ± 5,298	47,998 ± 4,712	52.6

[a]Percent inhibition of [^{3}H]leucine uptake was determined using the following formula: %I = [(mean cpm without ricin - mean cpm with ricin)/(mean cpm without ricin)] x 100.
[b]The final concentration of ricin used was 6.0ng/ml.
[c]Mean cpm of triplicate determinations.

RICIN A-18 PEPTIDE

SEQUENCE:

N-Terminus-C-V-Q-S-Y-T-N-F-I-R-A-V-R-L-T-T-G-A-D-V-R-COOH-Terminus

AMINO ACID ANALYSIS:

Asp	1	Leu	1
Asn	1	Nle	0
Thr	3	Tyr	1
Ser	1	Phe	1
Glu	0	His	0
Gly	2	Orn	0
Ala	2	Lys	0
Cys	1	Arg	4
Val	3	Pro	0
Met	0	Trp	0
Ile	1		

CHARACTERISTICS:

Molecular Weight:	2584.3
Extinction Coefficient:	0.6
Isoelectric Point:	9.2

Fig. 1. The amino acid sequence and characteristics of ricin A-18 synthetic peptide.

RICIN A-95 PEPTIDE

SEQUENCE:

N-Terminus-C-P-D-N-Q-E-D-A-E-A-I-T-H-L-F-T-D-V-Q-N-R-Y-T-F-A-F-G-

COOH-Terminus

AMINO ACID ANALYSIS:

Asp	3	Leu	1
Asn	2	Nle	0
Thr	3	Tyr	1
Ser	0	Phe	3
Glu	2	His	1
Gln	2	Pap	0
Gly	1	Orn	0
Ala	3	Lys	0
Cys	1	Arg	1
Val	1	Pro	1
Met	0	Trp	0
Ile	1		

CHARACTERISTICS:

Molecular Weight:	3103.7
Extinction Coefficient:	0.6
Isoelectric Point:	5.4

Fig. 2. The amino acid sequence and characteristics of ricin A-95 synthetic peptide.

RICIN B-18 PEPTIDE

SEQUENCE:

N-Terminus-G-L-C-V-D-V-R-D-G-R-F-H-N-G-N-A-I-Q-L-W-P-C-K-S-N-T-D-A-N-

Q-L-G-G-COOH-Terminus

AMINO ACID ANALYSIS:

Asp	3	Leu	3
Asn	4	Nle	0
Thr	1	Tyr	0
Ser	1	Phe	1
Glu	0	His	1
Gln	2	Pap	0
Gly	5	Orn	0
Ala	2	Lys	1
Cys	2	Arg	2
Val	2	Pro	1
Met	0	Trp	1
Ile	1		

CHARACTERISTICS:

Molecular Weight:	3557.4
Extinction Coefficient:	1.6
Isoelectric Point:	7.3

Fig. 3. The amino acid sequence and characteristics of ricin B-18 synthetic peptide.

RICIN B-230 PEPTIDE

SEQUENCE:

N-terminus-C-G-L-V-L-D-V-R-R-S-D-P-S-L-K-Q-I-I-L-Y-P-L-Y-P-L-H-G-D-P-

N-Q-G-COOH-Terminus

AMINO ACID ANALYSIS:

Asp	3	Leu	5
Asn	1	Nle	0
Thr	0	Tyr	1
Ser	2	Phe	0
Glu	0	His	0
Gln	2	Phe	0
Gly	3	Orn	0
Ala	0	Lys	1
Cys	1	Arg	2
Val	2	Pro	3
Met	0	Trp	0
Ile	2		

CHARACTERISTICS:

Molecular Weigth:	3205.1
Extinction Coefficient:	0.44
Isoelectric Point:	7.1

Fig. 4. The amino acid sequence and characteristics of ricin B-230 synthetic peptide.

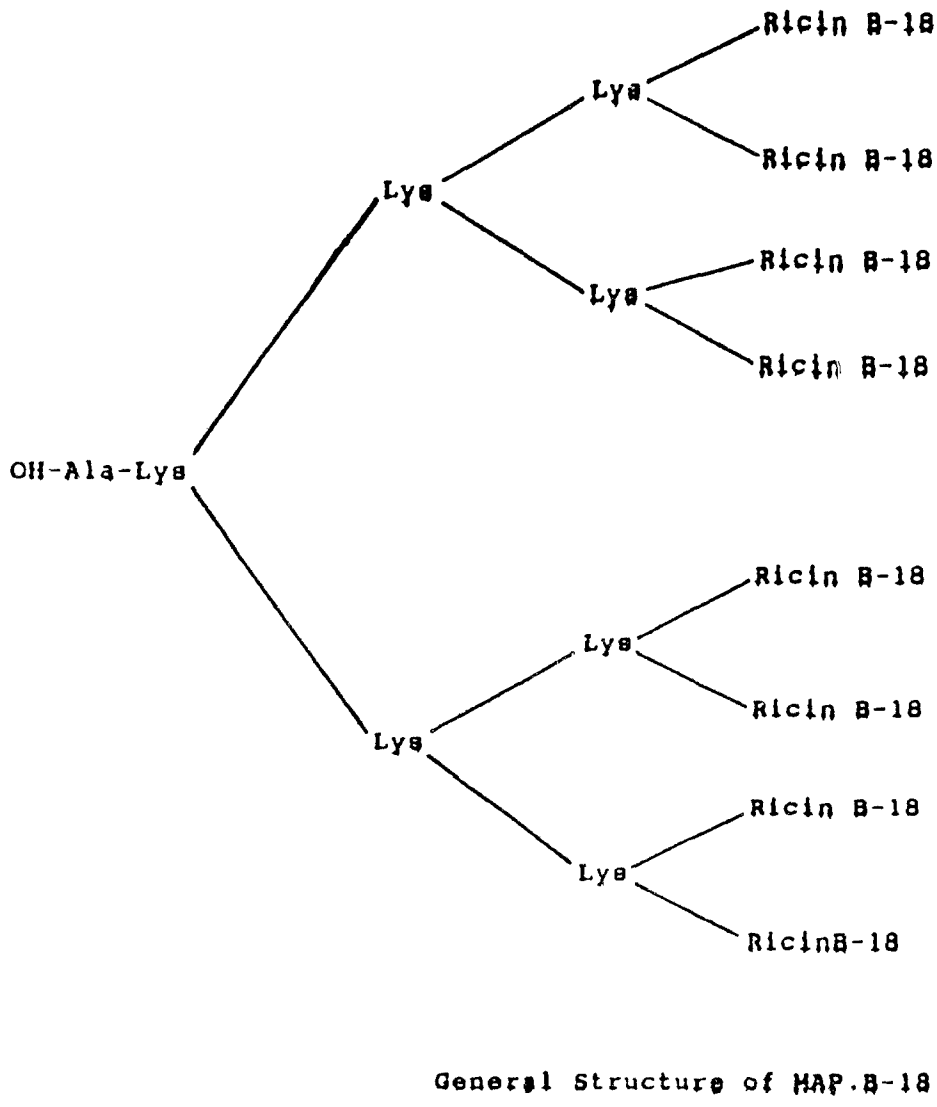

Fig. 5. The structure of ricin B-18 synthetic peptide on MAP.

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